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A pH-responsive cyclodextrin-based hybrid nanosystem as a nonviral vector for gene delivery

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ABSTRACT

The absence of safe, efficient, cost-effective, and easily scalable delivery platforms is one of the most significant hurdles and critical issues that limit the bench to bedside translation of oligonucleotides-based therapeutics. Acid-labile materials are of special interest in developing nonviral vectors due to their capability of intracellularly delivering therapeutic payload. In this study, a nanovector was designed by integrating a pH-responsive cyclodextrin material and low molecular weight polyethylenimine (PEI). Antisense oligonucleotide (ASON) Bcl-xl could be encapsulated into this hybrid nanosystem with extremely high loading efficiency by a nanoemulsion technique. The developed pH-responsive ASON nanotherapeutics could be efficiently transfected into human lung adenocarcinoma cells in a time- and dose-dependent manner, resulting in effective cell growth inhibition, significant suppression on the expression of Bcl-xl mRNA/protein, and efficient cell apoptosis. Importantly, the new nanovector showed drastically higher efficacy and lower cytotoxicity when compared with PLGA-based counterpart and commonly used cationic vectors like branched PEI (25,000 Da) and Lipofectamine 2000. This pH-responsive hybrid nanosystem may serve as a safe and efficient nonviral vector that may find wide applications in gene therapy.

1. Introduction

Exploring efficient nonviral vectors with excellent biocompatibility is still one of the most important topics in multidisciplinary fields of materials science, chemistry, biology, medicine, and pharmaceutical sciences [1–3]. The condensation and delivery of functional genes have been achieved using a plethora of cationic lipids and polymers, with the transfection efficiency closely related to the molecular structure of vectors used [1–6]. Alternatively, their encapsulation or loading and intracellular ferrying can be realized by various micro- or nanostructures including microcapsules [7], minicells [8], liposomes [9], lipid nanoparticles (NPs) [10], polymer NPs [11], inorganic NPs [12,13], and DNA/RNA assemblies [14,15]. A variety of gene therapeutics like plasmid DNA (pDNA) [16], antisense DNA/RNA oligonucleotides, small interfering RNA (siRNA) [17], microRNA (miRNA) [18], short hairpin RNA (shRNA) [19], and double-stranded RNA (dsRNA) [20], have been examined in these studies. In the case of antisense oligonucleotides (ASONs), they have been investigated to treat a variety of diseases including various cancers, diabetes, amyotrophic lateral sclerosis, Duchenne muscular dystrophy, and inflammatory diseases such as asthma and arthritis [21]. Whereas a series of cationic materials and polymer micro- or nanoparticles have been developed for both *in vitro* and *in vivo* transfection of ASONs [22–24], only limited success has been achieved so far. For the polycations-based transfection, condensation is generally implemented by complexation between negatively charged nucleic acids and polycationic molecules. The resulting polyplexes or lipoplexes formed by electrostatic forces may be easily destabilized by polyanionic biomolecules, which are abundant in blood and extracellular fluid [25]. In addition, inefficient condensation may occur due to the rigidity and low charge density of individual ASON, especially in the case of low molecular weight (Mw) polycations. Although this issue can be partly circumvented by using polycations with high charge density

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and high Mw, this strategy generally leads to increased cytotoxicity, tissue injury, or thrombus formation [25–29]. On the other hand, encapsulation of ASONs with polymers or lipids often results in low loading efficiency due to their high water-solubility and low surface charge of individual oligonucleotide [30,31]. As a result, desirable encapsulation remains particularly challenging. Deficiency of safe, efficient, cost-effective, and easily scalable delivery platforms is one of the most significant hurdles and critical issues that limit the bench to bedside translation of oligonucleotides-based therapeutics.

Materials that can undergo pH-sensitive degradation or hydrolysis are of special interest for selectively delivering therapeutics to subcellular organelles, tissues, or organs [32]. Given the low pH values in tumor tissue (pH <6.5), infectious and inflammatory sites (pH ~6.5) as well as in endosomal/lysosomal compartments (pH 4–5), low pH-triggered delivery systems have been extremely widely implemented to achieve better efficacy and avoid side effects [33–35]. The acid-labile nanosystems have been particularly employed for intracellular delivery of various therapeutics varying from small molecules, peptides, to proteins and nucleic acids, owing to their capability of releasing cargo molecules in endosomes to avoid transport to harmful lysosomes where degradation or inactivation generally occurs [36–39]. Nevertheless, development of acid-labile nanocarriers with excellent biocompatibility, controlled biodegradability, high payload loading capability, and fine-tuned cargo release kinetics, remains challenging with respect to gene delivery.

By kinetically controlled acetalation of cyclodextrins (CDs) and their polymers, recently we developed a series of pH-responsive materials that can be hydrolyzed under mildly acidic conditions [40]. Extensive *in vitro* and *in vivo* evaluation indicated that these materials exhibit excellent biocompatibility, making them suitable for drug delivery by oral, mucosal, subcutaneous, intramuscular, and intravenous administration. We hypothesize that a nonviral nanovector can be engineered by hybridizing a pH-sensitive acetalated α-CD material (Ac-aCD) and branched low Mw polyethylenimine (PEI) with Mw of 1800 (PEI1800), which can be easily produced by a modified nanoemulsion technique. PEI1800 is introduced to improve oligonucleotide transfection by increasing payload stability, enhancing loading efficiency, and facilitating endosomal escape. The utilization of Ac-aCD that can hydrolyze under mildly acidic conditions promises the payload release subsequent to transport via the endosomal pathway. Since Ac-aCD and PEI1800 are materials with low cytotoxicity, thus developed nanoplatform would be safe for *in vitro* and *in vivo* transfection. The aim of this study was to substantiate the construction of this pH-responsive nanovector and investigate its *in vitro* transfection capability, by using an ASON that targets to Bcl-xl, an antiapoptotic member of the Bcl-2 family which play a central role in cell apoptosis and proliferation, and are implicated in the pathology of many malignancies.

2. Materials and methods

2.1. Materials

Branched polyethylenimines with Mw of 1800 Da (PEI1800) or 25,000 Da (PEI25000) and poly(D,L-lactide-co-glycolide) [50:50] (PLGA) with intrinsic viscosity of 0.50–0.65 were purchased from Polysciences, Inc. (USA). 2-methoxypropene (MP) and α-cyclodextrin (α-CD) were obtained from Sigma (USA). Pyridinium *p*-toluene sulfonate (PTS) and poly(vinyl alcohol) (PVA) (88 mol% hydrolyzed, Mw = 25 kDa) were obtained from Acro Organics. Human lung adenocarcinomic cell line A549 was donated from Institute of Respiratory Diseases, Xinqiao Hospital of Third Military Medical University. RPMI-1640 medium, trypsin, and fetal bovine serum (FBS) were purchased from HyClone (USA). Bcl-xl antisense oligonucleotide (ASON) (5′ TCCCGGTTGCTCTGAGACAT 3′) and Cy3-labeled ASON (ASONCy3) at 5′ were synthesized by Sangon Biological Engineering Co., Ltd (Shanghai, China). Lipofectamine 2000 was purchased from Invitrogen. Antibodies against Bcl-xl from Abcam plc (Cambridge, UK) were used for Western blot assay. Secondary antibodies of peroxidase conjugated affinipure rabbit anti-goat IgG, goat anti-rat or mouse IgG and goat anti-mouse IgG were purchased from Beijing Golden Bridge Biotech (Beijing, China). Reverse transcription polymerase chain reaction (RT-PCR) kit was purchased from TaKaRa Bio, Inc. (Dalian, China). CellTiter 96 AQueous One Solution Cell Proliferation Assay was obtained from Promega (Madison, WI). All the other reagents are commercially available and used as received.

2.2. Synthesis of pH-responsive α-cyclodextrin material

α-CD was acetalated to produce pH-responsive material according to our previous study (Scheme 1A) [40]. Briefly, 4 mL MP (42 mmol) was added into 20 mL anhydrous DMSO containing 1 g α-CD (1.03 mmol), into which 16 mg PTS was added. After 3 h of acetonation, 0.5 mL of triethylamine was added into the reaction mixture to terminate the reaction. The acetalated product was precipitated from water, collected by filtration, thoroughly washed with water and lyophilized to a white powder of acetalated α-CD (Ac-aCD).

2.3. Fabrication of Bcl-xl ASON-containing nanovectors

A modified water-in-oil-in-water (W1/O/W2) double nanoemulsion method was adopted to fabricate Bcl-xl antisense loaded NPs [41]. For this purpose, 100 μL of ASON solution containing 1.0 nmol ASON was emulsified into 1.0 mL dichloromethane (DCM) containing 50 mg Ac-aCD and appropriate amount of PEI1800 by probe sonication. The obtained water-in-oil (W1/O) nanoemulsion was emulsified into 6 mL of 1.0% PVA aqueous solution to produce W1/O/W2 double nanoemulsion, which was then immediately poured into 20 mL of 0.3% PVA solution. After 3 h of magnetic stirring at room temperature, the solidified NPs were harvested by centrifugation at 16,000 rpm, and then rinsed four times with deionized water. ASON-containing PEI1800/PLGA hybrid NPs were prepared by the similar procedures.

The loading content of ASON in various NPs was determined by fluorescence measurement of ASONCy3. To this end, Ac-aCD NPs were thoroughly hydrolyzed in PBS buffer with pH 5. The fluorescence intensity of ASONCy3 in the aqueous solution was then measured by fluorescence spectroscopy (Perkin–Elmer LS55). The excitation wavelength was set at 550 nm, while the emission wavelength was 570 nm. The concentration of ASONCy3 was calculated according to a standard curve established with a series of ASONCy3 aqueous solutions with predetermined concentrations.

In the case of nanocomplexes derived from cationic materials, they were produced by directly mixing ASON solution with the aqueous solution containing PEI1800, PEI25000, or Lipofectamine 2000 at predetermined formulations. For PEI1800 and PEI25000, the N/P (molar ratio of total N in the cationic molecule to the total P in ASON) ratio was 10:1. The standard protocols provided by the manufacturer were adopted for Lipofectamine 2000. It should be noted that DNAase-free water was used to prepare aqueous solutions for the fabrication of both hybrid NPs and nanocomplexes in order to prevent the nuclease degradation of ASON.

2.4. In vitro release study

In vitro release kinetics of ASON-containing hybrid NPs based on either Ac-aCD or PLGA were studied under mildly acidic (pH 5) or physiological conditions (pH 7.4). Briefly, 10 mg of freshly fabricated NPs containing 400 pmol of ASONCy3 was dispersed in 1.5 mL PBS buffer with pH 5 or pH 7.4, which were incubated at 37 °C. At appropriate intervals, 1.0 mL of supernatant was withdrawn after centrifugation at 16,000 rpm, and the same volume of fresh medium was supplemented. The released ASONCy3 was quantified by fluorescence spectroscopy as described above.

2.5. In vitro degradation study

In vitro degradation of ASON-containing NPs derived from Ac-aCD or PLGA was performed at 37 °C in PBS buffers at pH 5 or pH 7.4. For this purpose, appropriate amount of freshly prepared NPs was dispersed in PBS buffers with pH 5 or pH 7.4, respectively. At various time points, the transmittance was measured at 500 nm. Digital photos were also taken at specific time points to give intuitive results.

2.6. Measurements

FT-IR spectrum was recorded on a Perkin–Elmer FT-IR spectrometer (100S). ^{1}H NMR spectrum was recorded on a Varian INOVA-400 spectrometer operating at 400 MHz. Dynamic light scattering (DLS) and ζ-potential measurements of various NPs in aqueous solution were performed on a Malvern Zetasizer Nano ZS instrument at 25 °C. Transmission electron microscopy (TEM) observation was carried out on a TECNAI-10 microscope (Philips, The Netherlands) operating at an acceleration voltage of 80 kV. Scanning electron microscopy (SEM) images were taken on an S-3400N II electron microscope (Hitachi, Japan).